EXHIBIT B2

6d ET-1 Stimulation of 20' FM2030

- 2.5 - 5 x 10^6 cells / 150cm² were seeded onto dishes
- cells were grown in [illegible] BU x [illegible] days prior to exp.
- cells fed c̄ media s̄ phorbol ester for duration of exp. ("no PMA")
- Media Δ QOD, [ET-1] & [ET3] = 10nM unless otherwise noted
- 100nM BQ123 or BQ788 added 1hr before exp. ET addition
- Herbimycin @ 400nM (1/2 recommended conc.) added to cells the day before ET addition → [↓] 2° to toxicity

150 cm² Dishes

Protein

10nM ET-1 1d
2d
3d*
4d*
5d
6d

~~No ET (6d)~~
~~1d~~
~~2d~~

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150cm² dishes

Protein

No ET	6d
10nM ET-1	1d
	2d
	3d*
	4d*
	5d
	6d
0.5nM ET-1	6d
1.0nM ET-1	6d
~~Keto BQ123 10nM 6d~~	

B

BQ123/10nM ET-1	4d
BQ123 alone	4d
BQ788/10nM ET-1	4d
BQ788 alone	4d
Kerbamycin/10nM ET-1	1d
Kerbamycin alone	1d
10nM ET-3	6d

RNA

No ET	
10nM ET-1	1d
	2d
	3d
	4d
	5d
	7d

100cm² dishes

Protein

No ET	1d
	2d
	3d*
	4d*
	5d

Cyt Fractions	OD_{595} (1λ) unless otherwise noted	γ/λ	5γ	20γ
1d NOET	0.142	1.8	2.78	11.11 ⇒ 13.3 (2.2)
• 1d + ET	0.200	2.2	2.27	9.09 ⇒ 10.9 (1.8)
2d NOET	0.148		1.82	7.27 ⇒ 8.7 (1.5)
• 2d + ET	0.234	2.75		
3d NOET	* .073 (2λ)			
• 3d + ET	* 0.126	1.0	5.0 *	20 ⇒ 24 (4)
4d NO ET	.082 (2λ)	0.9	5.56	22.22 ⇒ 26.7 (4.4)
• 4d + ET	.176 (2λ)			
5d NO ET	.103 (2λ)			
• 5d + ET	0.170	1.7	2.94	11.76 ⇒ 14.11 (2.4)
• 6d NO ET	0.109 (2λ)	0.45	11.11	44.44
• 6d + ET	0.198 (2λ)	1.1	4.55	18.18 ⇒ 21.8 (3.6)
6d 0.5nM ET	0.175 (2λ)			
6d 1.0nM ET	0.249 (2λ)			
6d ET3	0.285 (2λ)			
4d BQ123/NOET	0.126 (2λ)			
4d BQ788/NOET	0.163 (2λ)			
4d BQ123/ET A	0.275 (2λ)			
4d BQ123/ET B	0.163 (2λ)			
4d BQ123/ET C	0.235 (2λ)			
4d BQ123/ET D	0.206 (2λ)			
1d Herb/NOET	0.147	1.4		
1d Herb/ET	0.176	1.8		

Gel ① - ECAD cyt 20γ

1dNO - ~~6dNO~~ - 1(t) - 2(t) - 3(t) - 4(t) - 5(t) - 6(t)

Gel ② - βCAT cyt 5γ

3λ + 27λ 1X

5/1/6

x̄	
3dET	~~5.6/4.4/6.7~~
4dET	5.6/1.1/6.7
6dET	4.6/0.9/5.5

1dNO - 6dNO - 1(t) - 2(t) - 3(t) - 4(t) - 5(t) - 6(t)

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PM Fractions

		OD415(17)		2.5γ	25γ
2)	1d −	.115	1.0	2.5	
1.8)	· 1d +	.228	2.6	0.96	
	2d −	.152	1.5	1.67	
1.5)	· 2d +	.575	6.7	0.37	
	3d −	.086/.215(27)	0.7/1.2	2.08	
1)	· 3d +	.267	3.2	0.78	
	· 4d −	.115/.233(27)	0.9/1.3	~~2.78~~ 1.92	
1.4)	· 4d +	.428	5.5	0.45	
	5d −	.165	1.7	~~2.57~~ 1.47	
4)	5d +	.274	3.3	0.76	
	· 6d −	.202	2.25	1.11	11.1/2.2/13.3
3.6)	· 6d +	.296	3.6	0.69	6.9/1.4/8.3
	6d 0.5	.227	2.5	1.0	
	6d 1.0	.270	3.2	0.78	
ET3	4d [illegible]	.357	4.4	0.57	
	· 4d 780/−	.279	3.4	0.74	
	· 4d 123/+ A	.476	6.2	0.40	
	B	.223	2.5	1.0	
	C	.406	5.1	0.49	
	D	.309	3.7	0.68	
	1d Hab /−	.137	1.3	1.92	
	1d Hab +	.175	1.75	1.43	
	· 4d123 /−	.239	2.7	0.93	

Gel ③ ECAD PM

6dNO − 1(+) − 2(+) − 3(+) − 4(+) − 5(+) − 6(+)

3λ + 27 → 1X

Gel ④ Antag. Prelim.

3λ + 27 → 1X 4dNo − 4d(+) − 123(−) − 780(−) − 123(+) A − 123(+) B − 12 C

Gel ⑤ Titration, ET3

No − 0.5 − 1.0 − 6d(+) − ET3 3λ → 27 → 1X

6d.

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Gel (6) : NoET, Herb

Herb / Kerb, 1(+), 1(-), 2(-) 3(-), 4(-), 6(-)

(2) 6 (3/4) (5) (6)

Gel (7) - MCAM, ICBAM, (C) 44, MCAM
2.5

(PM) ~~6(-) 35λ + 7λ 6X, 3λ + 27λ 1X~~

~~6(+) 25λ + 5λ 6X, 3λ + 27λ 1X~~

6(-) 25λ + 5λ 6X — 4 - 20/4λ ⇒ 23

6(+) 20λ + 4λ 6X — 4 + 50/1λ ⇒ 5.4

Repeat : Run both gels longer

Gel (2) : BCAT at 5λ
3λ → 12λ 1X

1no-	1+	2+	3+	4+	5+	6+
13.9	11.35	9.1	12	13.4	14.7	11.2

Gel (4) : ~~Probe c~~ Antisense probe c ECAD + BCAT sequential

4d NO / 4d + / 123 - / (E) / 788 / (B)

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Results

Ecad:
PM

Duration: ECAD ↓ beginning @ day 1 c̄ almost complete shutoff day 5. "Blip" ↑ on day 5 * c̄ decrease well below control sample day 6.

* on day 4, ET-1 readdition occurred after midnight ie: put 48hr mark

Antagonists: 123/ET and 788/ET Not all labeled "123/ET" labeled them A-D. A+C show ↓ ECAD expression: will assume that ~~these~~ these are 123/ET samples. B+D show ECAD expression similar to baseline ∴ will assume that these are 788/ET samples ∴ samples re-run

If above assumptions are correct BQ123 does not antagonize ~~EO~~ ECAD ↓ induced by ET but BQ788 does ~~so~~ ∅ inhibit ET-induced downregulation of ECAD

Titration Time point 6d

ET-1 @ 0.5nM c̄ ~3X ↓ ECAD. Similar ↓ @ 1.0nM
~6X ↓ reg @ 10nM

ET3 10nM
~10X ↓ reg of ECAD day 6

Herbimycin 1d timepoint
- Herbimycin inhibits ↓ ECAD induced by ET
- very toxic to cells

No ET
↓ in ECAD during course of experiment but @ day 1, 4 and 6, ET stimulated samples all c̄ relative ↓ in ECAD expression. Day 2, 3, 5 not tested.

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BCAT cyt

No ECAD in cyt. day 1. Appearance of ECAD in cyt. day 2-6

BCAT PM

↑ in mobility and ↓ protein day 2 and 4.

* ? no Δ mobility, day 6 because of late addition day 4?

BCAT antagonist day 4

mobility shift inhibited by 788 but not 123

BCAT pool BCAT cyt

↑ mobility day 2, 5, 6

ICAM-1, CD44, NCAD

GT-1 effect

MCAM

Potent GT-1 induced down regulation

~~Conclu~~

Conclusions

1) ET-1 decreases ECAD protein over 6d time course in late passage (20 FM2030) melanocytes.
2) This effect is mediated by the ETRB subtype
3) ET-1 Rx causes the appearance of cytoplasmic ECAD beginning day 2
4) Herbamycin inhibits ET induced ↓ in ECAD as day 1 ... Tyr. kinase may be required for same
5) ET1 induced ↓ ECAD is dose responsive and can be noted at concentration as low as 0.5nM
6) ET3, which is selective for ETRB is a more potent ↓ reg. of ECAD than ET-1
7) ET-1 induces an increase in mobility of melanocytes. BCAT and a decrease in amt of membrane assoc. BCAT ~~48 hrs~~ between 24 & 48 hrs of ET-1 stimulation
8) ET-1 causes amobility shift in cytoplasmic BCAT as well.
9) ET-1 is a potent downreg. of MCAM
10) ET-1 has no effect on ICAM-1, CD44, and VCAM expression.

OVER

BCAD

Future experiments

1) Will determine whether ET-1 induces (P) Tyr ing βCAT

2) Will attempt to see if mobility shift due to dephosphorylation (?? inhibition of GSK-3)

3) Will detach cells for mono to plating g c̄ Versene instead of trypsin which may be responsible for ↓ in ECAD seen in unstimulated samples (ie: trypsinization may cleave ECAD, resulting in an ↑ in expression followed by gradual ↓ to baseline)

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